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(21) International Application Number: PCT/US95/06260 (22) International Filing Date: 6 June 1995 (06.06.95) (30) Priority Data: 08/294,251 23 August 1994 (23.08.94) US (60) Parent Application or Grant (63) Related by Continuation US 08/294,251 (CIP) Filed on 23 August 1994 (23.08.94) (71) Applicant (for all designated States except US): HUMAN GENOME SCIENCES, INC. [US/US]; 9410 Key West Avenue, Rockville, MD 20850-3338 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): LI, Haodong [-/US]; 11033 Rutledge Drive, Gaithersburg, MD 20878 (US). ADAMS, Mark, D. [-/US]; 15205 Dufief Drive, North Potomac, MD 20878 (US).		(74) Agents: OLSTEIN, Elliot, M.; Carella, Byrne, Bain, Gilfillan, Cecchi, Stewart & Olstein, 6 Becker Farm Road, Roseland, NJ 07068 (US) et al. (81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).  <b>Published</b> With international search report.	
(54) Title: HUMAN CHEMOKINE BETA-9			
(57) Abstract  Human Ck $\beta$ -9 polypeptides and DNA (RNA) encoding such chemokine polypeptides and a procedure for producing such polypeptides by recombinant techniques is disclosed. Also disclosed are methods for utilizing such Ck $\beta$ -9 polypeptides for the treatment of leukemia, tumors, chronic infections, autoimmune diseases, fibrotic disorders, wound healing and psoriasis. Antagonists against such polypeptides and their use as a therapeutic agent to treat rheumatoid arthritis, autoimmune and chronic inflammatory and infective diseases, allergic reactions, prostaglandin-independent fever and bone marrow failure are also disclosed. Diagnostic assays are also disclosed which detect the presence of mutations in the Ck $\beta$ -9 coding sequence and over-expression of the Ck $\beta$ -9 protein.			

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### Human Chemokine Beta-9

This invention relates to newly identified polynucleotides, polypeptides encoded by such polynucleotides, the use of such polynucleotides and polypeptides, as well as the production of such polynucleotides and polypeptides. More particularly, the polypeptides of the present invention are human chemokine beta-9 sometimes hereinafter referred to as "Ck $\beta$ -9". The invention also relates to inhibiting the action of such polypeptides.

Chemokines, also referred to as intercrine cytokines, are a subfamily of structurally and functionally related cytokines. These molecules are 8-10 kd in size. In general, chemokines exhibit 20% to 75% homology at the amino acid level and are characterized by four conserved cysteine residues that form two disulfide bonds. Based on the arrangement of the first two cysteine residues, chemokines have been classified into two subfamilies, alpha and beta. In the alpha subfamily, the first two cysteines are separated by one amino acid and hence are referred to as the "C-X-C" subfamily. In the beta subfamily, the two cysteines are in an adjacent position and are, therefore, referred to as the "C-C" subfamily. Thus far, at least eight different members of this family have been identified in humans.

The intercrine cytokines exhibit a wide variety of functions. A hallmark feature is their ability to elicit chemotactic migration of distinct cell types, including monocytes, neutrophils, T lymphocytes, basophils and fibroblasts. Many chemokines have pro-inflammatory activity and are involved in multiple steps during an inflammatory reaction. These activities include stimulation of histamine release, lysosomal enzyme and leukotriene release, increased adherence of target immune cells to endothelial cells, enhanced binding of complement proteins, induced expression of granulocyte adhesion molecules and complement receptors, and respiratory burst. In addition to their involvement in inflammation, certain chemokines have been shown to exhibit other activities. For example, macrophage inflammatory protein 1 (MIP-1) is able to suppress hematopoietic stem cell proliferation, platelet factor-4 (PF-4) is a potent inhibitor of endothelial cell growth, Interleukin-3 (IL-3) promotes proliferation of keratinocytes, and GRO is an autocrine growth factor for melanoma cells.

In light of the diverse biological activities, it is not surprising that chemokines have been implicated in a number of physiological and disease conditions, including lymphocyte trafficking, wound healing, hematopoietic regulation and immunological disorders such as allergy, asthma and arthritis.

Members of the "C-C" branch exert their effects on the following cells: eosinophils which destroy parasites to lessen parasitic infection and cause chronic inflammation in the airways of the respiratory system; macrophages which suppress tumor formation in vertebrates; and basophils which release histamine which plays a role in allergic inflammation. However, members of one branch may exert an effect on cells which are normally responsive to the other branch of chemokines and, therefore, no precise role can be attached to the members of the branches.

While members of the C-C branch act predominantly on mononuclear cells and members of the C-X-C branch act predominantly on neutrophils a distinct chemoattractant property cannot be assigned to a chemokine based on this guideline. Some chemokines from one family show characteristics of the other.

The polypeptide of the present invention has been putatively identified as Ck $\beta$ -9 based on amino acid sequence homology.

In accordance with one aspect of the present invention, there are provided novel polypeptides as well as biologically active and diagnostically or therapeutically useful fragments, analogs and derivatives thereof. The polypeptide of the present invention is of human origin.

In accordance with another aspect of the present invention, there are provided isolated nucleic acid molecules encoding the polypeptide of the present invention, including mRNAs, DNAs, cDNAs, genomic DNAs as well as analogs and biologically active and diagnostically or therapeutically useful fragments and derivatives thereof.

In accordance with yet a further aspect of the present invention, there is provided a process for producing such polypeptides by recombinant techniques comprising culturing recombinant prokaryotic and/or eukaryotic host cells, containing a nucleic acid sequence encoding a polypeptide of the present invention, under conditions promoting expression of said protein and subsequent recovery of said protein.

In accordance with yet a further aspect of the present invention, there is provided a process for utilizing such polypeptides, or polynucleotides encoding such polypeptides for therapeutic purposes, for example, to treat solid tumors, chronic infections, auto-immune diseases, psoriasis, asthma, allergy, to regulate hematopoiesis, and to promote wound healing.

In accordance with yet a further aspect of the present invention, there are provided antibodies against such polypeptides.

In accordance with yet another aspect of the present invention, there are provided antagonists to such polypeptides, which may be used to inhibit the action of such polypeptides, for example, in the treatment of auto-immune diseases, chronic inflammatory diseases, histamine-mediated allergic reactions, asthma, arthritis, prostaglandin-independent fever, bone marrow failure, silicosis, sarcoidosis, hyper-eosinophilic syndrome and lung inflammation.

In accordance with yet a further aspect of the present invention, there are also provided nucleic acid probes comprising nucleic acid molecules of sufficient length to specifically hybridize to a nucleic acid sequence of the present invention.

In accordance with still another aspect of the present invention, there are provided diagnostic assays for detecting diseases related to the expression of the polypeptide and mutations in the nucleic acid sequences encoding such polypeptide.

In accordance with yet a further aspect of the present invention, there is provided a process for utilizing such polypeptides, or polynucleotides encoding such polypeptides, for *in vitro* purposes related to scientific research, synthesis of DNA and manufacture of DNA vectors.

These and other aspects of the present invention should be apparent to those skilled in the art from the teachings herein.

The following drawings are illustrative of embodiments of the invention and are not meant to limit the scope of the invention as encompassed by the claims.

Figure 1 displays the cDNA sequence and corresponding deduced amino acid sequence of Ck $\beta$ -9. The initial 23 amino

acids represent the leader sequence such that the putative mature polypeptide comprises 111 amino acids. The standard one-letter abbreviation for amino acids is used.

Figure 2 displays the amino acid sequence homology between Ck $\beta$ -9 and the mature peptide of eotaxin (bottom).

In accordance with an aspect of the present invention, there are provided isolated nucleic acids (polynucleotides) which encode for the mature polypeptides having the deduced amino acid sequences of Figure 1 (SEQ ID No. 2) or for the mature polypeptide encoded by the cDNA of the clones deposited as ATCC Deposit No. 75803 on June 7, 1994.

The polynucleotide encoding Ck $\beta$ -9 was discovered in a cDNA library derived from a human breast lymph node. Ck $\beta$ -9 is structurally related to the chemokine family. It contains an open reading frame encoding a protein of 134 amino acid residues of which approximately the first 23 amino acids residues are the putative leader sequence such that the mature protein comprises 111 amino acids. The protein exhibits the highest degree of homology to eotaxin with 32% identity and 69% similarity over a stretch of 75 amino acid residues. It is also important that the four spatially conserved cysteine residues in chemokines are found in the polypeptides of the present invention.

The polynucleotides of the present invention may be in the form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. The coding sequence which encodes the mature polypeptides may be identical to the coding sequence shown in Figure 1 (SEQ ID No. 1) or that of the deposited clones or may be a different coding sequence which coding sequence, as a result of the redundancy or degeneracy of the genetic code, encodes the same mature polypeptides as the DNA of Figure 1 (SEQ ID No. 1) or the deposited cDNA.

The polynucleotides which encodes for the mature polypeptides of Figure 1 (SEQ ID No. 2) or for the mature polypeptides encoded by the deposited cDNA may include: only the coding sequence for the mature polypeptide; the coding sequence for the mature polypeptide and additional coding sequence such as a leader or secretory sequence or a proprotein sequence; the coding sequence for the mature polypeptide (and optionally additional coding sequence) and non-coding sequence, such as introns or non-coding sequence 5' and/or 3' of the coding sequence for the mature polypeptides.

Thus, the term "polynucleotide encoding a polypeptide" encompasses a polynucleotide which includes only coding sequence for the polypeptide as well as a polynucleotide which includes additional coding and/or non-coding sequence.

The present invention further relates to variants of the hereinabove described polynucleotides which encode for fragments, analogs and derivatives of the polypeptide having the deduced amino acid sequence of Figure 1 (SEQ ID No. 2) or the polypeptide encoded by the cDNA of the deposited clones. The variant of the polynucleotides may be a naturally occurring allelic variant of the polynucleotides or a non-naturally occurring variant of the polynucleotides.

Thus, the present invention includes polynucleotides encoding the same mature polypeptides as shown in Figure 1 (SEQ ID No. 2) or the same mature polypeptides encoded by the cDNA of the deposited clones as well as variants of such polynucleotides which variants encode for a fragment, derivative or analog of the polypeptides of Figure 1 (SEQ ID No. 2) or the polypeptides encoded by the cDNA of the deposited clones. Such nucleotide variants include deletion variants, substitution variants and addition or insertion variants.

As hereinabove indicated, the polynucleotides may have a coding sequence which is a naturally occurring allelic



variant of the coding sequence shown in Figure 1 (SEQ ID No. 2) or of the coding sequence of the deposited clones. As known in the art, an allelic variant is an alternate form of a polynucleotide sequence which may have a substitution, deletion or addition of one or more nucleotides, which does not substantially alter the function of the encoded polypeptide.

The present invention also includes polynucleotides, wherein the coding sequence for the mature polypeptides may be fused in the same reading frame to a polynucleotide sequence which aids in expression and secretion of a polypeptide from a host cell, for example, a leader sequence which functions as a secretory sequence for controlling transport of a polypeptide from the cell. The polypeptide having a leader sequence is a preprotein and may have the leader sequence cleaved by the host cell to form the mature form of the polypeptide. The polynucleotides may also encode for a proprotein which is the mature protein plus additional 5' amino acid residues. A mature protein having a prosequence is a proprotein and is an inactive form of the protein. Once the prosequence is cleaved an active mature protein remains.

Thus, for example, the polynucleotide of the present invention may encode for a mature protein, or for a protein having a prosequence or for a protein having both a prosequence and a presequence (leader sequence).

The polynucleotides of the present invention may also have the coding sequence fused in frame to a marker sequence which allows for purification of the polypeptides of the present invention. The marker sequence may be a hexahistidine tag supplied by a pQE-9 vector to provide for purification of the mature polypeptides fused to the marker in the case of a bacterial host, or, for example, the marker sequence may be a hemagglutinin (HA) tag when a mammalian host, e.g. COS-7 cells, is used. The HA tag corresponds to

an epitope derived from the influenza hemagglutinin protein (Wilson, I., et al., Cell, 37:767 (1984)).

The term "gene" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons).

Fragments of the full length gene of the present invention may be used as a hybridization probe for a cDNA library to isolate the full length cDNA and to isolate other cDNAs which have a high sequence similarity to the gene or similar biological activity. Probes of this type preferably have at least 30 bases and may contain, for example, 50 or more bases. The probe may also be used to identify a cDNA clone corresponding to a full length transcript and a genomic clone or clones that contain the complete gene including regulatory and promotor regions, exons, and introns. An example of a screen comprises isolating the coding region of the gene by using the known DNA sequence to synthesize an oligonucleotide probe. Labeled oligonucleotides having a sequence complementary to that of the gene of the present invention are used to screen a library of human cDNA, genomic DNA or mRNA to determine which members of the library the probe hybridizes to.

The present invention further relates to polynucleotides which hybridize to the hereinabove-described sequences if there is at least 70%, preferably at least 90%, and more preferably at least 95% identity between the sequences. The present invention particularly relates to polynucleotides which hybridize under stringent conditions to the hereinabove-described polynucleotides. As herein used, the term "stringent conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences. The polynucleotides which hybridize to the hereinabove described polynucleotides

in a preferred embodiment encode polypeptides which either retain substantially the same biological function or activity as the mature polypeptide encoded by the cDNAs of Figure 1 (SEQ ID NO:1) or the deposited cDNA(s).

Alternatively, the polynucleotide may have at least 20 bases, preferably 30 bases, and more preferably at least 50 bases which hybridize to a polynucleotide of the present invention and which has an identity thereto, as hereinabove described, and which may or may not retain activity. For example, such polynucleotides may be employed as probes for the polynucleotide of SEQ ID NO:1, for example, for recovery of the polynucleotide or as a diagnostic probe or as a PCR primer.

Thus, the present invention is directed to polynucleotides having at least a 70% identity, preferably at least 90% and more preferably at least a 95% identity to a polynucleotide which encodes the polypeptide of SEQ ID NO:2 as well as fragments thereof, which fragments have at least 30 bases and preferably at least 50 bases and to polypeptides encoded by such polynucleotides.

The deposit(s) referred to herein will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for purposes of Patent Procedure. These deposits are provided merely as convenience to those of skill in the art and are not an admission that a deposit is required under 35 U.S.C. §112. The sequence of the polynucleotides contained in the deposited materials, as well as the amino acid sequence of the polypeptides encoded thereby, are incorporated herein by reference and are controlling in the event of any conflict with any description of sequences herein. A license may be required to make, use or sell the deposited materials, and no such license is hereby granted.

The present invention further relates to chemokine polypeptides which have the deduced amino acid sequences of

Figure 1 (SEQ ID No. 2) or which has the amino acid sequence encoded by the deposited cDNA, as well as fragments, analogs and derivatives of such polypeptides.

The terms "fragment," "derivative" and "analog" when referring to the polypeptides of Figure 1 (SEQ ID No. 2) or that encoded by the deposited cDNA, means polypeptides which retain essentially the same biological function or activity as such polypeptides. Thus, an analog includes a proprotein which can be activated by cleavage of the proprotein portion to produce an active mature polypeptide.

The chemokine polypeptides of the present invention may be recombinant polypeptides, natural polypeptides or a synthetic polypeptides, preferably recombinant polypeptides.

The fragment, derivative or analog of the polypeptides of Figure 1 (SEQ ID No. 2) or that encoded by the deposited cDNA may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature polypeptide, such as a leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

The polypeptides and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

The polypeptides of the present invention include the polypeptide of SEQ ID NO:2 (in particular the mature polypeptide) as well as polypeptides which have at least 70% similarity (preferably at least 70% identity) to the polypeptide of SEQ ID NO:2 and more preferably at least 90% similarity (more preferably at least 90% identity) to the polypeptide of SEQ ID NO:2 and still more preferably at least 95% similarity (still more preferably at least 95% identity) to the polypeptide of SEQ ID NO:2 and also include portions of such polypeptides with such portion of the polypeptide generally containing at least 30 amino acids and more preferably at least 50 amino acids.

As known in the art "similarity" between two polypeptides is determined by comparing the amino acid sequence and its conserved amino acid substitutes of one polypeptide to the sequence of a second polypeptide.

Fragments or portions of the polypeptides of the present invention may be employed for producing the corresponding full-length polypeptide by peptide synthesis; therefore, the fragments may be employed as intermediates for producing the full-length polypeptides. Fragments or portions of the polynucleotides of the present invention may be used to synthesize full-length polynucleotides of the present invention.

The present invention also relates to vectors which include polynucleotides of the present invention, host cells which are genetically engineered with vectors of the invention and the production of polypeptides of the invention by recombinant techniques.

Host cells are genetically engineered (transduced or transformed or transfected) with the vectors of this invention which may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the  $Ck\beta$ -9 genes. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The polynucleotides of the present invention may be employed for producing polypeptides by recombinant techniques. Thus, for example, the polynucleotide may be included in any one of a variety of expression vectors for expressing a polypeptide. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences, e.g., derivatives of SV40; bacterial plasmids; phage DNA; baculovirus; yeast plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other vector may be used as long as it is replicable and viable in the host.

The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art.

The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. As representative examples of such promoters, there may be mentioned: LTR or SV40 promoter, the E. coli lac or trp, the phage lambda P<sub>i</sub> promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression.

In addition, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in E. coli.

The vector containing the appropriate DNA sequence as hereinabove described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein.

As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as E. coli, Streptomyces, Salmonella typhimurium; fungal cells, such as yeast; insect cells such as Drosophila S2 and Spodoptera Sf9; animal cells such as CHO, COS or Bowes melanoma; adenoviruses; plant cells, etc. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

More particularly, the present invention also includes recombinant constructs comprising one or more of the sequences as broadly described above. The constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this

embodiment, the construct further comprises regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example. Bacterial: pQE70, pQE60, pQE-9 (Qiagen), pBS, pD10, phagescript, psiX174, pBluescript SK, pBSKS, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene); ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia). Eukaryotic: pWLNEO, pSV2CAT, pOG44, pXT1, pSG (Stratagene) pSVK3, pBPV, pMSG, pSVL (Pharmacia). However, any other plasmid or vector may be used as long as they are replicable and viable in the host.

Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are pKK232-8 and pCM7. Particular named bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda P<sub>R</sub>, P<sub>L</sub> and trp. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art.

In a further embodiment, the present invention relates to host cells containing the above-described constructs. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation (Davis, L., Dibner, M., Battey, I., Basic Methods in Molecular Biology, (1986)).

The constructs in host cells can be used in a conventional manner to produce the gene product encoded by



the recombinant sequence. Alternatively, the polypeptides of the invention can be synthetically produced by conventional peptide synthesizers.

Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor, N.Y., (1989), the disclosure of which is hereby incorporated by reference.

Transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp that act on a promoter to increase its transcription. Examples including the SV40 enhancer on the late side of the replication origin bp 100 to 270, a cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, e.g., the ampicillin resistance gene of *E. coli* and *S. cerevisiae* TRP1 gene, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK),  $\alpha$ -factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or

extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product.

Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transformation include E. coli, Bacillus subtilis, Salmonella typhimurium and various species within the genera Pseudomonas, Streptomyces, and Staphylococcus, although others may also be employed as a matter of choice.

As a representative but nonlimiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and pGEM1 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is induced by appropriate means (e.g., temperature shift or chemical induction) and cells are cultured for an additional period.

Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents, such methods are well known to those skilled in the art.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, Cell, 23:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

The Ck $\beta$ -9 polypeptides can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

The polypeptides of the present invention may be a naturally purified product, or a product of chemical synthetic procedures, or produced by recombinant techniques

from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture). Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. Polypeptides of the invention may also include an initial methionine amino acid residue.

The Ck $\beta$ -9 polypeptides may be employed to inhibit bone marrow stem cell colony formation as adjunct protective treatment during cancer chemotherapy and for leukemia.

The chemokine polypeptides may also be used to inhibit epidermal keratinocyte proliferation for treatment of psoriasis, which is characterized by keratinocyte hyperproliferation.

The chemokine polypeptides may also be used to treat solid tumors by stimulating the invasion and activation of host defense cells, e.g., cytotoxic T cells and macrophages. They may also be used to enhance host defenses against resistant chronic infections, for example, mycobacterial infections via the attraction and activation of microbicidal leukocytes.

The chemokine polypeptides may also be used to treat auto-immune disease and lymphocytic leukemia by inhibiting T cell proliferation by the inhibition of IL2 biosynthesis.

Ck $\beta$ -9 may also be used in wound healing, both via the recruitment of debris clearing and connective tissue promoting inflammatory cells and also via its control of excessive TGF $\beta$ -mediated fibrosis. In this same manner, Ck $\beta$ -9 may also be used to treat other fibrotic disorders, including liver cirrhosis, osteoarthritis and pulmonary fibrosis.

The chemokine polypeptides also increase the presence of eosinophils which have the distinctive function of killing the larvae of parasites that invade tissues, as in schistosomiasis, trichinosis and ascariasis.

They may also be used to regulate hematopoiesis, by regulating the activation and differentiation of various hematopoietic progenitor cells, for example, to release mature leukocytes from the bone marrow following chemotherapy.

The polynucleotides and polypeptides encoded by such polynucleotides may also be utilized for *in vitro* purposes related to scientific research, synthesis of DNA and manufacture of DNA vectors and for designing therapeutics and diagnostics for the treatment of human disease.

Fragments of the full length Ck $\beta$ -9 genes may be used as a hybridization probe for a cDNA library to isolate the full length gene and to isolate other genes which have a high sequence similarity to the gene or similar biological activity. Probes of this type can be, for example, between 20 and 2000 bases. Preferably, however, the probes have between 30 and 50 base pairs. The probe may also be used to identify a cDNA clone corresponding to a full length transcript and a genomic clone or clones that contain the complete genes including regulatory and promotor regions, exons, and introns. An example of a screen comprises isolating the coding region of the genes by using the known DNA sequence to synthesize an oligonucleotide probe. Labeled oligonucleotides having a sequence complementary to that of the genes of the present invention are used to screen a library of human cDNA, genomic DNA or mRNA to determine which members of the library the probe hybridizes to.

This invention is also related to the use of the Ck $\beta$ -9 gene as part of a diagnostic assay for detecting diseases or susceptibility to diseases related to the presence of mutations in the Ck $\beta$ -9 nucleic acid sequences. Such diseases are related to under-expression of the chemokine polypeptides, for example, tumors and cancers.

Individuals carrying mutations in the Ck $\beta$ -9 gene may be detected at the DNA level by a variety of techniques.

Nucleic acids for diagnosis may be obtained from a patient's cells, such as from blood, urine, saliva, tissue biopsy and autopsy material. The genomic DNA may be used directly for detection or may be amplified enzymatically by using PCR (Saiki et al., Nature, 324:163-166 (1986)) prior to analysis. RNA or cDNA may also be used for the same purpose. As an example, PCR primers complementary to the nucleic acid encoding Ck $\beta$ -9 can be used to identify and analyze Ck $\beta$ -9 mutations. For example, deletions and insertions can be detected by a change in size of the amplified product in comparison to the normal genotype. Point mutations can be identified by hybridizing amplified DNA to radiolabeled Ck $\beta$ -9 RNA or alternatively, radiolabeled Ck $\beta$ -9 antisense DNA sequences. Perfectly matched sequences can be distinguished from mismatched duplexes by RNase A digestion or by differences in melting temperatures.

Genetic testing based on DNA sequence differences may be achieved by detection of alteration in electrophoretic mobility of DNA fragments in gels with or without denaturing agents. Small sequence deletions and insertions can be visualized by high resolution gel electrophoresis. DNA fragments of different sequences may be distinguished on denaturing formamide gradient gels in which the mobilities of different DNA fragments are retarded in the gel at different positions according to their specific melting or partial melting temperatures (see, e.g., Myers et al., Science, 230:1242 (1985)).

Sequence changes at specific locations may also be revealed by nuclease protection assays, such as RNase and S1 protection or the chemical cleavage method (e.g., Cotton et al., PNAS, USA, 85:4397-4401 (1985)).

Thus, the detection of a specific DNA sequence may be achieved by methods such as hybridization, RNase protection, chemical cleavage, direct DNA sequencing or the use of

restriction enzymes, (e.g., Restriction Fragment Length Polymorphisms (RFLP)) and Southern blotting of genomic DNA.

In addition to more conventional gel-electrophoresis and DNA sequencing, mutations can also be detected by *in situ* analysis.

The present invention also relates to a diagnostic assay for detecting altered levels of Ck $\beta$ -9 protein in various tissues since an over-expression of the proteins compared to normal control tissue samples may detect the presence of a disease or susceptibility to a disease, for example, a tumor. Assays used to detect levels of Ck $\beta$ -9 protein in a sample derived from a host are well-known to those of skill in the art and include radioimmunoassays, competitive-binding assays, Western Blot analysis, ELISA assays and "sandwich" assay. An ELISA assay (Coligan, et al., Current Protocols in Immunology, 1(2), Chapter 6, (1991)) initially comprises preparing an antibody specific to the Ck $\beta$ -9 antigen, preferably a monoclonal antibody. In addition a reporter antibody is prepared against the monoclonal antibody. To the reporter antibody is attached a detectable reagent such as radioactivity, fluorescence or, in this example, a horseradish peroxidase enzyme. A sample is removed from a host and incubated on a solid support, e.g. a polystyrene dish, that binds the proteins in the sample. Any free protein binding sites on the dish are then covered by incubating with a non-specific protein like bovine serum albumin. Next, the monoclonal antibody is incubated in the dish during which time the monoclonal antibodies attach to any Ck $\beta$ -9 proteins attached to the polystyrene dish. All unbound monoclonal antibody is washed out with buffer. The reporter antibody linked to horseradish peroxidase is now placed in the dish resulting in binding of the reporter antibody to any monoclonal antibody bound to Ck $\beta$ -9. Unattached reporter antibody is then washed out. Peroxidase substrates are then added to the dish and the amount of color

developed in a given time period is a measurement of the amount of Ck $\beta$ -9 protein present in a given volume of patient sample when compared against a standard curve.

A competition assay may be employed wherein antibodies specific to Ck $\beta$ -9 are attached to a solid support and labeled Ck $\beta$ -9 and a sample derived from the host are passed over the solid support and the amount of label detected, for example by liquid scintillation chromatography, can be correlated to a quantity of Ck $\beta$ -9 in the sample.

A "sandwich" assay is similar to an ELISA assay. In a "sandwich" assay Ck $\beta$ -9 is passed over a solid support and binds to antibody attached to a solid support. A second antibody is then bound to the Ck $\beta$ -9. A third antibody which is labeled and specific to the second antibody is then passed over the solid support and binds to the second antibody and an amount can then be quantified.

This invention provides a method for identification of the receptors for Ck $\beta$ -9 polypeptides. The gene encoding the receptor can be identified by numerous methods known to those of skill in the art, for example, ligand panning and FACS sorting (Coligan, et al., Current Protocols in Immun., 1(2), Chapter 5, (1991)). Preferably, expression cloning is employed wherein polyadenylated RNA is prepared from a cell responsive to the polypeptides, and a cDNA library created from this RNA is divided into pools and used to transfect COS cells or other cells that are not responsive to the polypeptides. Transfected cells which are grown on glass slides are exposed to the labeled polypeptides. The polypeptides can be labeled by a variety of means including iodination or inclusion of a recognition site for a site-specific protein kinase. Following fixation and incubation, the slides are subjected to autoradiographic analysis. Positive pools are identified and sub-pools are prepared and re-transfected using an iterative sub-pooling and re-



screening process, eventually yielding a single clones that encodes the putative receptor.

As an alternative approach for receptor identification, the labeled polypeptides can be photo-affinity linked with cell membrane or extract preparations that express the receptor molecule. Cross-linked material is resolved by PAGE analysis and exposed to x-ray film. The labeled complex containing the receptors of the polypeptides can be excised, resolved into peptide fragments, and subjected to protein micro-sequencing. The amino acid sequence obtained from micro-sequencing would be used to design a set of degenerate oligonucleotide probes to screen a cDNA library to identify the genes encoding the putative receptors.

This invention provides a method of screening compounds to identify agonists and antagonists to the Ck $\beta$ -9 polypeptides of the present invention. An agonist is a compound which has similar biological functions of the polypeptides, while antagonists block such functions. Chemotaxis may be assayed by placing cells, which are chemo-attracted by either of the polypeptides of the present invention, on top of a filter with pores of sufficient diameter to admit the cells (about 5  $\mu$ m). Solutions of potential agonists are placed in the bottom of the chamber with an appropriate control medium in the upper compartment, and thus a concentration gradient of the agonist is measured by counting cells that migrate into or through the porous membrane over time.

When assaying for antagonists, the polypeptides of the present invention are placed in the bottom chamber and the potential antagonist is added to determine if chemotaxis of the cells is prevented.

Alternatively, a mammalian cell or membrane preparation expressing the receptors of the polypeptides would be incubated with a labeled Ck $\beta$ -9 polypeptide, eg. radioactivity, in the presence of the compound. The ability

of the compound to block this interaction could then be measured. When assaying for agonists in this fashion, the chemokines would be absent and the ability of the agonist itself to interact with the receptor could be measured.

Examples of potential Ck $\beta$ -9 antagonists include antibodies, or in some cases, oligonucleotides, which bind to the polypeptides. Another example of a potential antagonist is a negative dominant mutant of the polypeptides. Negative dominant mutants are polypeptides which bind to the receptor of the wild-type polypeptide, but fail to retain biological activity.

Antisense constructs prepared using antisense technology are also potential antagonists. Antisense technology can be used to control gene expression through triple-helix formation or antisense DNA or RNA, both of which methods are based on binding of a polynucleotide to DNA or RNA. For example, the 5' coding portion of the polynucleotide sequence, which encodes for the mature polypeptides of the present invention, is used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription (triple-helix, see Lee et al., Nucl. Acids Res., 6:3073 (1979); Cooney et al, Science, 241:456 (1988); and Dervan et al., Science, 251:1360 (1991)), thereby preventing transcription and the production of the chemokine polypeptides. The antisense RNA oligonucleotide hybridizes to the mRNA *in vivo* and blocks translation of the mRNA molecule into the polypeptides (antisense - Okano, J. Neurochem., 56:560 (1991); Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988)). The oligonucleotides described above can also be delivered to cells such that the antisense RNA or DNA may be expressed *in vivo* to inhibit production of Ck $\beta$ -9.

Another potential Ck $\beta$ -9 antagonist is a peptide derivative of the polypeptides which are naturally or synthetically modified analogs of the polypeptides that have lost biological function yet still recognize and bind to the receptors of the polypeptides to thereby effectively block the receptors. Examples of peptide derivatives include, but are not limited to, small peptides or peptide-like molecules.

The antagonists may be employed to inhibit the chemotaxis and activation of macrophages and their precursors, and of neutrophils, basophils, B lymphocytes and some T cell subsets, e.g., activated and CD8 cytotoxic T cells and natural killer cells, in auto-immune and chronic inflammatory and infective diseases. Examples of auto-immune diseases include rheumatoid arthritis, multiple sclerosis, and insulin-dependent diabetes. Some infectious diseases include silicosis, sarcoidosis, idiopathic pulmonary fibrosis by preventing the recruitment and activation of mononuclear phagocytes, idiopathic hyper-eosinophilic syndrome by preventing eosinophil production and migration, endotoxic shock by preventing the migration of macrophages and their production of the chemokine polypeptides of the present invention.

The antagonists may also be employed for treating atherosclerosis, by preventing monocyte infiltration in the artery wall.

The antagonists may also be employed to treat histamine-mediated allergic reactions and immunological disorders including late phase allergic reactions, chronic urticaria, and atopic dermatitis by inhibiting chemokine-induced mast cell and basophil degranulation and release of histamine. IgE-mediated allergic reactions such as allergic asthma, rhinitis, and eczema may also be treated.

The antagonists may also be employed to treat chronic and acute inflammation by preventing the attraction of monocytes to a wound area. They may also be employed to

regulate normal pulmonary macrophage populations, since acute and chronic inflammatory pulmonary diseases are associated with sequestration of mononuclear phagocytes in the lung.

Antagonists may also be employed to treat rheumatoid arthritis by preventing the attraction of monocytes into synovial fluid in the joints of patients. Monocyte influx and activation plays a significant role in the pathogenesis of both degenerative and inflammatory arthropathies.

The antagonists may be employed to interfere with the deleterious cascades attributed primarily to IL-1 and TNF, which prevents the biosynthesis of other inflammatory cytokines. In this way, the antagonists may be employed to prevent inflammation. The antagonists may also be employed to inhibit prostaglandin-independent fever induced by chemokines.

The antagonists may also be employed to treat cases of bone marrow failure, for example, aplastic anemia and myelodysplastic syndrome.

The antagonists may also be used to treat asthma and allergy by preventing eosinophil accumulation in the lung.

The antagonists may also be employed to treat subepithelial basement membrane fibrosis which is a prominent feature of the asthmatic lung.

The antagonists may be employed in a composition with a pharmaceutically acceptable carrier, e.g., as hereinafter described.

The Ck $\beta$ -9 polypeptides and agonists and antagonists may be employed in combination with a suitable pharmaceutical carrier. Such compositions comprise a therapeutically effective amount of the polypeptide, and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The formulation should suit the mode of administration.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. In addition, the polypeptides and agonists and antagonists may be employed in conjunction with other therapeutic compounds.

The pharmaceutical compositions may be administered in a convenient manner such as by the topical, intravenous, intraperitoneal, intramuscular, intratumor, subcutaneous, intranasal or intradermal routes. The pharmaceutical compositions are administered in an amount which is effective for treating and/or prophylaxis of the specific indication. In general, the polypeptides will be administered in an amount of at least about 10  $\mu\text{g/kg}$  body weight and in most cases they will be administered in an amount not in excess of about 8 mg/Kg body weight per day. In most cases, the dosage is from about 10  $\mu\text{g/kg}$  to about 1 mg/kg body weight daily, taking into account the routes of administration, symptoms, etc.

The Ck $\beta$ -9 polypeptides, and agonists or antagonists which are polypeptides, may be employed in accordance with the present invention by expression of such polypeptides *in vivo*, which is often referred to as "gene therapy."

Thus, for example, cells from a patient may be engineered with a polynucleotide (DNA or RNA) encoding a polypeptide *ex vivo*, with the engineered cells then being provided to a patient to be treated with the polypeptide. Such methods are well-known in the art. For example, cells may be engineered by procedures known in the art by use of a retroviral particle containing RNA encoding a polypeptide of the present invention.

Similarly, cells may be engineered *in vivo* for expression of a polypeptide *in vivo* by, for example, procedures known in the art. As known in the art, a producer cell for producing a retroviral particle containing RNA encoding the polypeptide of the present invention may be administered to a patient for engineering cells *in vivo* and expression of the polypeptide *in vivo*. These and other methods for administering a polypeptide of the present invention by such method should be apparent to those skilled in the art from the teachings of the present invention. For example, the expression vehicle for engineering cells may be other than a retrovirus, for example, an adenovirus which may be used to engineer cells *in vivo* after combination with a suitable delivery vehicle.

The sequences of the present invention are also valuable for chromosome identification. The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. Moreover, there is a current need for identifying particular sites on the chromosome. Few chromosome marking reagents based on actual sequence data (repeat polymorphisms) are presently available for marking chromosomal location. The mapping of DNAs to chromosomes according to the present invention is an important first step in correlating those sequences with genes associated with disease.

Briefly, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the cDNA. Computer analysis of the 3' untranslated region is used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human gene corresponding to the primer will yield an amplified fragment.

PCR mapping of somatic cell hybrids is a rapid procedure for assigning a particular DNA to a particular chromosome. Using the present invention with the same oligonucleotide primers, sublocalization can be achieved with panels of fragments from specific chromosomes or pools of large genomic clones in an analogous manner. Other mapping strategies that can similarly be used to map to its chromosome include *in situ* hybridization, prescreening with labeled flow-sorted chromosomes and preselection by hybridization to construct chromosome specific-cDNA libraries.

Fluorescence *in situ* hybridization (FISH) of a cDNA clones to a metaphase chromosomal spread can be used to provide a precise chromosomal location in one step. This technique can be used with cDNA as short as 50 or 60 bases. For a review of this technique, see Verma et al., *Human Chromosomes: a Manual of Basic Techniques*, Pergamon Press, New York (1988).

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. Such data are found, for example, in V. McKusick, *Mendelian Inheritance in Man* (available on line through Johns Hopkins University Welch Medical Library). The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes).

Next, it is necessary to determine the differences in the cDNA or genomic sequence between affected and unaffected individuals. If a mutation is observed in some or all of the affected individuals but not in any normal individuals, then the mutation is likely to be the causative agent of the disease.

With current resolution of physical mapping and genetic mapping techniques, a cDNA precisely localized to a chromosomal region associated with the disease could be one

of between 50 and 500 potential causative genes. (This assumes 1 megabase mapping resolution and one gene per 20 kb).

The polypeptides, their fragments or other derivatives, or analogs thereof, or cells expressing them can be used as an immunogen to produce antibodies thereto. These antibodies can be, for example, polyclonal or monoclonal antibodies. The present invention also includes chimeric, single chain, and humanized antibodies, as well as Fab fragments, or the product of an Fab expression library. Various procedures known in the art may be used for the production of such antibodies and fragments.

Antibodies generated against the polypeptides corresponding to a sequence of the present invention can be obtained by direct injection of the polypeptides into an animal or by administering the polypeptides to an animal, preferably a nonhuman. The antibody so obtained will then bind the polypeptides itself. In this manner, even a sequence encoding only a fragment of the polypeptides can be used to generate antibodies binding the whole native polypeptides. Such antibodies can then be used to isolate the polypeptide from tissue expressing that polypeptide.

For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler and Milstein, 1975, *Nature*, 256:495-497), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, *Immunology Today* 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole, et al., 1985, in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96).

Techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce single chain antibodies to immunogenic polypeptide products of this invention. Also, transgenic mice may be used to



express humanized antibodies to immunogenic polypeptide products of this invention.

The present invention will be further described with reference to the following examples; however, it is to be understood that the present invention is not limited to such examples. All parts or amounts, unless otherwise specified, are by weight.

In order to facilitate understanding of the following examples certain frequently occurring methods and/or terms will be described.

"Plasmids" are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accord with published procedures. In addition, equivalent plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

"Digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For analytical purposes, typically 1  $\mu$ g of plasmid or DNA fragment is used with about 2 units of enzyme in about 20  $\mu$ l of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50  $\mu$ g of DNA are digested with 20 to 250 units of enzyme in a larger volume. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37°C are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion the reaction is

electrophoresed directly on a polyacrylamide gel to isolate the desired fragment.

Size separation of the cleaved fragments is performed using 8 percent polyacrylamide gel described by Goeddel, D. et al., Nucleic Acids Res., 8:4057 (1980).

"Oligonucleotides" refers to either a single stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands which may be chemically synthesized. Such synthetic oligonucleotides have no 5' phosphate and thus will not ligate to another oligonucleotide without adding a phosphate with an ATP in the presence of a kinase. A synthetic oligonucleotide will ligate to a fragment that has not been dephosphorylated.

"Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (Maniatis, T., et al., Id., p. 146). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units to T4 DNA ligase ("ligase") per 0.5  $\mu$ g of approximately equimolar amounts of the DNA fragments to be ligated.

Unless otherwise stated, transformation was performed as described in the method of Graham, F. and Van der Eb, A., Virology, 52:456-457 (1973).

#### Example 1

##### Bacterial Expression and Purification of Ck $\beta$ -9

The DNA sequence encoding for Ck $\beta$ -9, ATCC # 75803, is initially amplified using PCR oligonucleotide primers corresponding to the 5' and 3' end sequences of the processed Ck $\beta$ -9 gene (minus the putative signal peptide sequence). Additional nucleotides corresponding to Ck $\beta$ -9 were added to the 5' and 3' end sequences, respectively. The 5' oligonucleotide primer has the sequence 5' **CCCGCATGCGTGATGGAGGGGCTCAG** 3' (SEQ ID No. 3) contains a SphI restriction enzyme site (bold) followed by 17 nucleotides of

Ck $\beta$ -9 coding sequence (underlined) starting from the second nucleotide of the sequences coding for the mature protein. The ATG codon is included in the SphI site. In the next codon following the ATG, the first base is from the SphI site and the remaining two bases correspond to the second and third base of the first codon (residue S<sub>M</sub>) of the putative mature protein. As a consequence, the first base in this codon is changed from A to C comparing with the original sequences, resulting in an S to R substitution in the recombinant protein. The 3' sequence 5' AAAGGATCCTGGCCCTTT AGGGGTCTGTGA 3' (SEQ ID No. 4) contains complementary sequences to a BamHI site (bold) and is followed by 21 nucleotides of gene specific sequences preceding the termination codon. The restriction enzyme sites correspond to the restriction enzyme sites on the bacterial expression vector pQE-70 (Qiagen, Inc. Chatsworth, CA). pQE-70 encodes antibiotic resistance (Amp<sup>r</sup>), a bacterial origin of replication (ori), an IPTG-regulatable promoter operator (P/O), a ribosome binding site (RBS), a 6-His tag and restriction enzyme sites. pQE-70 is then digested with SphI and BamHI. The amplified sequences are ligated into pQE-9 and inserted in frame with the sequence encoding for the histidine tag and the RBS. The ligation mixture is then used to transform the E. coli strain M15/rep4 (Qiagen, Inc.) by the procedure described in Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, Cold Spring Laboratory Press, (1989). M15/rep4 contains multiple copies of the plasmid pREP4, which expresses the lacI repressor and also confers kanamycin resistance (Kan<sup>r</sup>). Transformants are identified by their ability to grow on LB plates and ampicillin/kanamycin resistant colonies are selected. Plasmid DNA is isolated and confirmed by restriction analysis. Clones containing the desired constructs are grown overnight (O/N) in liquid culture in LB media supplemented with both Amp (100 ug/ml) and Kan (25 ug/ml). The O/N culture is used to inoculate a

large culture at a ratio of 1:100 to 1:250. The cells are grown to an optical density 600 (O.D.<sup>600</sup>) of between 0.4 and 0.6. IPTG ("Isopropyl-B-D-thiogalacto pyranoside") is then added to a final concentration of 1 mM. IPTG induces by inactivating the lacI repressor, clearing the P/O leading to increased gene expression. Cells are grown an extra 3 to 4 hours. Cells are then harvested by centrifugation. The cell pellet is solubilized in the chaotropic agent 6 Molar Guanidine HCl pH 5.0. After clarification, solubilized Ck $\beta$ -9 is purified from this solution by chromatography on a Nickel-Chelate column under conditions that allow for tight binding by proteins containing the 6-His tag (Hochuli, E. et al., J. Chromatography 411:177-184 (1984)). Ck $\beta$ -9 (>98% pure) is eluted from the column in 6M guanidine HCl. Protein renaturation out of GnHCl can be accomplished by several protocols (Jaenicke, R. and Rudolph, R., Protein Structure - A Practical Approach, IRL Press, New York (1990)). Initially, step dialysis is utilized to remove the GnHCl. Alternatively, the purified protein isolated from the Ni-chelate column can be bound to a second column over which a decreasing linear GnHCl gradient is run. The protein is allowed to renature while bound to the column and is subsequently eluted with a buffer containing 250 mM Imidazole, 150 mM NaCl, 25 mM Tris-HCl pH 7.5 and 10% Glycerol. Finally, soluble protein is dialyzed against a storage buffer containing 5 mM Ammonium Bicarbonate.

#### Example 2

##### Expression of Recombinant Ck $\beta$ -9 in COS cells

The expression of plasmid, Ck $\beta$ -9 HA is derived from a vector pcDNAI/Amp (Invitrogen) containing: 1) SV40 origin of replication, 2) ampicillin resistance gene, 3) E.coli replication origin, 4) CMV promoter followed by a polylinker region, a SV40 intron and polyadenylation site. A DNA

fragment encoding the entire Ck $\beta$ -9 precursor and a HA tag fused in frame to its 3' end is cloned into the polylinker region of the vector, therefore, the recombinant protein expression is directed under the CMV promoter. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein as previously described (I. Wilson, H. Niman, R. Heighten, A. Cherenson, M. Connolly, and R. Lerner, 1984, Cell 37, 767). The infusion of HA tag to the target protein allows easy detection of the recombinant protein with an antibody that recognizes the HA epitope.

The plasmid construction strategy is described as follows:

The DNA sequence encoding for Ck $\beta$ -9, ATTC # 75803, is constructed by PCR using two primers: the 5' primer 5' AAAGGATCCAGACATGGCTCAGTCACT 3' (SEQ ID No. 5) contains a BamHI site followed by 18 nucleotides of Ck $\beta$ -9 coding sequence starting from the initiation codon; the 3' sequence 5' CGCTCTAGATCAAGCGTAGTCTGGGACGTCGTATGGGTATGGCCCTTTAGGGGTCTG 3' (SEQ ID No. 6) contains complementary sequences to XbaI site, translation stop codon, HA tag and the last 18 nucleotides of the Ck $\beta$ -9 coding sequence (not including the stop codon). Therefore, the PCR product contains a BamHI site, Ck $\beta$ -9 coding sequence followed by HA tag fused in frame, a translation termination stop codon next to the HA tag, and an XbaI site. The PCR amplified DNA fragment and the vector, pcDNA1/Amp, are digested with BamHI and XbaI restriction enzyme and ligated. The ligation mixture is transformed into E. coli strain SURE (Stratagene Cloning Systems, La Jolla, CA) the transformed culture is plated on ampicillin media plates and resistant colonies are selected. Plasmid DNA is isolated from transformants and examined by restriction analysis for the presence of the correct fragment. For expression of the recombinant Ck $\beta$ -9, COS cells are transfected with the expression vector by DEAE-Dextran method (J. Sambrook, E. Fritsch, T. Maniatis, Molecular

Cloning: A Laboratory Manual, Cold Spring Laboratory Press, (1989)). The expression of the Ck $\beta$ -9 HA protein is detected by radiolabelling and immunoprecipitation method (E. Harlow, D. Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, (1988)). Cells are labelled for 8 hours with <sup>35</sup>S-cysteine two days post transfection. Culture media is then collected and cells are lysed with detergent (RIPA buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 1% NP-40, 0.5% DOC, 50mM Tris, pH 7.5) (Wilson, I. et al., Id. 37:767 (1984))). Both cell lysate and culture media are precipitated with a HA specific monoclonal antibody. Proteins precipitated are analyzed by SDS-PAGE.

### Example 3

#### Expression via Gene Therapy

Fibroblasts are obtained from a subject by skin biopsy. The resulting tissue is placed in tissue-culture medium and separated into small pieces. Small chunks of the tissue are placed on a wet surface of a tissue culture flask, approximately ten pieces are placed in each flask. The flask is turned upside down, closed tight and left at room temperature over night. After 24 hours at room temperature, the flask is inverted and the chunks of tissue remain fixed to the bottom of the flask and fresh media (e.g., Ham's F12 media, with 10% FBS, penicillin and streptomycin, is added. This is then incubated at 37°C for approximately one week. At this time, fresh media is added and subsequently changed every several days. After an additional two weeks in culture, a monolayer of fibroblasts emerge. The monolayer is trypsinized and scaled into larger flasks.

pmV-7 (Kirschmeier, P.T. et al, DNA, 7:219-25 (1988) flanked by the long terminal repeats of the Moloney murine sarcoma virus, is digested with EcoRI and HindIII and subsequently treated with calf intestinal phosphatase. The

linear vector is fractionated on agarose gel and purified, using glass beads.

The cDNA encoding a polypeptide of the present invention is amplified using PCR primers which correspond to the 5' and 3' end sequences respectively. The 5' primer containing an EcoRI site and the 3' primer further includes a HindIII site. Equal quantities of the Moloney murine sarcoma virus linear backbone and the amplified EcoRI and HindIII fragment are added together, in the presence of T4 DNA ligase. The resulting mixture is maintained under conditions appropriate for ligation of the two fragments. The ligation mixture is used to transform bacteria HB101, which are then plated onto agar-containing kanamycin for the purpose of confirming that the vector had the gene of interest properly inserted.

The amphotropic pA317 or GP+aml2 packaging cells are grown in tissue culture to confluent density in Dulbecco's Modified Eagles Medium (DMEM) with 10% calf serum (CS), penicillin and streptomycin. The MSV vector containing the gene is then added to the media and the packaging cells are transduced with the vector. The packaging cells now produce infectious viral particles containing the gene (the packaging cells are now referred to as producer cells).

Fresh media is added to the transduced producer cells, and subsequently, the media is harvested from a 10 cm plate of confluent producer cells. The spent media, containing the infectious viral particles, is filtered through a millipore filter to remove detached producer cells and this media is then used to infect fibroblast cells. Media is removed from a sub-confluent plate of fibroblasts and quickly replaced with the media from the producer cells. This media is removed and replaced with fresh media. If the titer of virus is high, then virtually all fibroblasts will be infected and no selection is required. If the titer is very low, then it is necessary to use a retroviral vector that has a selectable marker, such as neo or his.

The engineered fibroblasts are then injected into the host, either alone or after having been grown to confluence on cytodex 3 microcarrier beads. The fibroblasts now produce the protein product.

Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, within the scope of the appended claims, the invention may be practiced otherwise than as particularly described.



## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: LI, ET AL.
- (ii) TITLE OF INVENTION: Human Chemokine Beta-9
- (iii) NUMBER OF SEQUENCES: 6
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: CARELLA, BYRNE, BAIN, GILFILLAN,  
CECCHI, STEWART & OLSTEIN
  - (B) STREET: 6 BECKER FARM ROAD
  - (C) CITY: ROSELAND
  - (D) STATE: NEW JERSEY
  - (E) COUNTRY: USA
  - (F) ZIP: 07068
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: 3.5 INCH DISKETTE
  - (B) COMPUTER: IBM PS/2
  - (C) OPERATING SYSTEM: MS-DOS
  - (D) SOFTWARE: WORD PERFECT 5.1
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE: Concurrently
  - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:

- (viii) ATTORNEY/AGENT INFORMATION:
- (A) NAME: FERRARO, GREGORY D.
  - (B) REGISTRATION NUMBER: 36,134
  - (C) REFERENCE/DOCKET NUMBER: 325800-434
- (ix) TELECOMMUNICATION INFORMATION:
- (A) TELEPHONE: 201-994-1700
  - (B) TELEFAX: 201-994-1744
- (2) INFORMATION FOR SEQ ID NO:1:
- (i) SEQUENCE CHARACTERISTICS
- (A) LENGTH: 405 BASE PAIRS
  - (B) TYPE: NUCLEIC ACID
  - (C) STRANDEDNESS: SINGLE
  - (D) TOPOLOGY: LINEAR
- (ii) MOLECULE TYPE: cDNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGGCTCAGT	CACTGGCTCT	GAGCCTCCTT	ATCCTGGTTC	TGGCCTTTGG	CATCCCCAGG	60
ACCCAAGGCA	GTGATGGAGG	GGCTCAGGAC	TGTTGCCTCA	AGTACAGCCA	AAGGAAGATT	120
CCCGCCAAGG	TTGTCCGCAG	CTACCGGAAG	CAGGAACCAA	GCTTAGGCTG	CTCCATCCCA	180
GCTATCCTGT	TCTTGCCCCG	CAAGCGCTCT	CAGGCAGAGC	TATGTGCAGA	CCCAAAGGAG	240
CTCTGGGTGC	AGCAGCTGAT	GCAGCATCTG	GACAAGACAC	CATCCCCACA	GAAACCAGCC	300
CAGGGCTGCA	GGAAGGACAG	GGGGGCCTCC	AAGACTGGCA	AGAAAGGAAA	GGGCTCCAAA	360
GGCTGCAAGA	GGACTGAGCG	GTCACAGACC	CCTAAAGGGC	CATAG		405

## (3) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 134 AMINO ACIDS  
 (B) TYPE: AMINO ACID  
 (C) STRANDEDNESS:  
 (D) TOPOLOGY: LINEAR

## (ii) MOLECULE TYPE: PROTEIN

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met	Ala	Gln	Ser	Leu	Ala	Leu	Ser	Leu	Leu	Ile	Leu	Val	Leu	Ala	-20	-15	-10
Phe	Gly	Ile	Pro	Arg	Thr	Gln	Gly	Ser	Asp	Gly	Gly	Ala	Gln	Asp	-5	1	5
Cys	Cys	Leu	Lys	Tyr	Ser	Gln	Arg	Lys	Ile	Pro	Ala	Lys	Val	Val	10	15	20
Arg	Ser	Tyr	Arg	Lys	Gln	Glu	Pro	Ser	Leu	Gly	Cys	Ser	Ile	Pro	25	30	35
Ala	Ile	Leu	Phe	Leu	Pro	Arg	Lys	Arg	Ser	Gln	Ala	Glu	Leu	Cys	40	45	50
Ala	Asp	Pro	Lys	Glu	Leu	Tyr	Val	Gln	Gln	Leu	Met	Gln	His	Leu	55	60	65
Asp	Lys	Thr	Pro	Ser	Pro	Gln	Lys	Pro	Ala	Gln	Gly	Cys	Arg	Lys	70	75	80
Asp	Arg	Gly	Ala	Ser	Lys	Thr	Gly	Lys	Lys	Gly	Lys	Gly	Ser	Lys	85	90	95
Gly	Cys	Lys	Arg	Thr	Glu	Arg	Ser	Gln	Thr	Pro	Lys	Gly	Pro		100	105	110

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 26 BASE PAIRS

- (B) TYPE: NUCLEIC ACID
- (C) STRANDEDNESS: SINGLE
- (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCCGCATGCG TGATGGAGGG GCTCAG

26

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS
  - (A) LENGTH: 30 BASE PAIRS
  - (B) TYPE: NUCLEIC ACID
  - (C) STRANDEDNESS: SINGLE
  - (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AAAGGATCCT GGCCCTTTAG GGGTCTGTGA

30

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS
  - (A) LENGTH: 27 BASE PAIRS
  - (B) TYPE: NUCLEIC ACID
  - (C) STRANDEDNESS: SINGLE
  - (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AAAGGATCCA GACATGGCTC AGTCACT

27

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 56 BASE PAIRS

(B) TYPE: NUCLEIC ACID

(C) STRANDEDNESS: SINGLE

(D) TOPOLOGY: LINEAR

## (ii) MOLECULE TYPE: Oligonucleotide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CGCTCTAGAT CAAGCGTAGT CTGGGACGTCG TATGGGTATG GCCCTTTAGG GGTCTG 56

WHAT IS CLAIMED IS:

1. An isolated polynucleotide comprising a member selected from the group consisting of:
  - (a) a polynucleotide encoding the polypeptide comprising amino acid -23 to amino acid 111 as set forth in SEQ ID NO:2;
  - (b) a polynucleotide encoding the polypeptide comprising amino acid 1 to amino acid 111 as set forth in SEQ ID NO:2
  - (c) a polynucleotide capable of hybridizing to and which is at least 70% identical to the polynucleotide of (a) or (b); and
  - (d) a polynucleotide fragment of the polynucleotide of (a), (b) or (c).
2. The polynucleotide of Claim 1 wherein the polynucleotide is DNA.
3. The polynucleotide of Claim 2 which encodes the polypeptide comprising amino acid 1 to 111 of SEQ ID NO:2.
4. An isolated polynucleotide comprising a member selected from the group consisting of:
  - (a) a polynucleotide which encodes a mature polypeptide having the amino acid sequence expressed by the DNA contained in ATCC Deposit No. 75803;
  - (b) a polynucleotide which encodes a polypeptide having the amino acid sequence expressed by the DNA contained in ATCC Deposit No. 75803;
  - (c) a polynucleotide capable of hybridizing to and which is at least 70% identical to the polynucleotide of (a); and
  - (d) a polynucleotide fragment of the polynucleotide of (a), (b) or (c).

5. The polynucleotide of claim 1 comprising the sequence as set forth in SEQ ID No. 1 from nucleotide 1 to nucleotide 405.
6. The polynucleotide of claim 1 comprising the sequence as set forth in SEQ ID No. from nucleotide 70 to nucleotide 405.
7. A vector containing the DNA of Claim 2.
8. A host cell genetically engineered with the vector of Claim 7.
9. A process for producing a polypeptide comprising: expressing from the host cell of Claim 8 the polypeptide encoded by said DNA.
10. A process for producing cells capable of expressing a polypeptide comprising genetically engineering cells with the vector of Claim 7.
11. A polypeptide encoded by the polynucleotide of claim 1 comprising a member selected from the group consisting of (i) a mature polypeptide having the deduced amino acid sequence of SEQ ID NO:2 and fragments, analogs and derivatives thereof; and (ii) a mature polypeptide encoded by the cDNA of ATCC Deposit No. 75803 and fragments, analogs and derivatives of said polypeptide.
12. The polypeptide of Claim 11 wherein the polypeptide comprises amino acid 1 to amino acid 111 of SEQ ID NO:2.
13. A compound which inhibits activation of the receptor for the polypeptide of claim 11.

14. A compound which activates the receptor for the polypeptide of claim 11.
15. A method for the treatment of a patient having need of Ck $\beta$ -9 comprising: administering to the patient a therapeutically effective amount of the polypeptide of claim 11.
16. The method of Claim 15 wherein said therapeutically effective amount of the polypeptide is administered by providing to the patient DNA encoding said polypeptide and expressing said polypeptide *in vivo*.
17. A method for the treatment of a patient having need to inhibit a Ck $\beta$ -9 polypeptide comprising: administering to the patient a therapeutically effective amount of the compound of Claim 13.
18. A process for diagnosing a disease or a susceptibility to a disease related to an under-expression of the polypeptide of claim 11 comprising:
  - determining a mutation in a nucleic acid sequence encoding said polypeptide.
19. A diagnostic process comprising:
  - analyzing for the presence of the polypeptide of claim 11 in a sample derived from a host.
20. A method for identifying agonist or antagonist compounds to the polypeptide of claim 11 comprising:
  - contacting a cell expressing on the surface thereof a receptor for the polypeptide, said receptor being associated with a second component capable of providing a detectable signal in response to the binding of a compound



to said receptor, with an analytically detectable compound under conditions to permit binding to the receptor;

detecting the absence or presence of a signal generated from the interaction of the compound with the receptor.

1 / 2

## FIG. 1

ATGGCTCAGTCACTGGCTCTGAGCCTCCTTATCCTGGTCTGGCCTTGGCATCCCCAGG  
M A Q S L A L S L L I L V L A F G I P R

ACCCAAGCAGTGATGGAGGGCTCAGGACTGTTGCCCTCAAGTACAGCCAAGGAAGATT  
T Q G S D G G A Q D C C L K Y S Q R K I

CCCCCAAGGTGTCCGCAGCTACCGGAAGCAGGAACCAAGCTTAGGCTGCTCCATCCCA  
P A K V V R S Y R K Q E P S L G C S I P

GCTATCCTGTTCTTGCCCCGCAAGCGCTCTCAGGCAGAGCTATGTGCAGACCCAAAGGAG  
A I L F L P R K R S Q A E L C A D P K E

CTCTGGTGTCAGCAGCTGATGCAGCATCTGGACAAGACACCATCCCCACAGAAACAGCC  
L W V Q Q L M Q H L D K T P S P Q K P A

CAGGGCTGCAGGAAGGACAGGGGGCCTCCAAGACTGGCAAGAAAGGGCTCCAAA  
Q G C R K D R G A S K T G K K G K G S K

GGCTGCAAGAGGACTGACGGGTCACAGACCCCTAAAGGGCCATAG  
G C K R T E R S Q T P K G P \*

2 / 2

## FIG. 2

25 D G G A Q D C C L K Y S Q . R K I P A K V V R S Y R K Q E P S L G C S I P A I L F L P R K R S Q A E 73  
 . | : . | | : : . . | | . . . : | | . . | | . . | | : | : . :  
 2 P G I P S A C C F R V T N I C K I S F Q A L K S Y . K I I T S S K C P Q T A I V F . . E I K P D K H 48  
 74 L C A D P K E L W V Q Q L M Q H L D K T P S P Q K P 99  
 : | | | : | | | : . . . | | . . . | |  
 49 I C A D P R X G W V Q D A K K Y L D Q I S Q X T K P 74

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/06260

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.5; 435/69.5, 320.1, 240.2, 252.3, 254.11, 7.21, 29; 530/351, 395; 424/85.2; 514/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Sequence databases: GenBank/EMBL/DDBJ, GeneSeq

Keyword databases: Medline, Biosis, SciSearch, CAS, USPTO-APS

Search terms: chemokine, beta[9], eotaxin

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Journal of Experimental Medicine, Volume 179, issued March 1994, P. J. Jose et al., "Eotaxin: A Potent Eosinophil Chemoattractant Cytokine Detected in a Guinea Pig Model of Allergic Airways Inflammation", pages 881-887.	1-12, 14-16, 18, 19
A,P	Biochemical and Biophysical Research Communications, Volume 205, Number 1, issued 30 November 1994, P.J. Jose et al., "Eotaxin: cloning of an eosinophil chemoattractant cytokine and increased mRNA expression in allergen-challenged guinea-pig lungs", pages 788-794.	1-12, 14-16, 18, 19

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"A" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

08 AUGUST 1995

Date of mailing of the international search report

28 AUG 1995

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
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Washington, D.C. 20231

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/06260

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	Journal of Experimental Medicine, Volume 181, issued March 1995, M. E. Rothenberg et al., "Constitutive and Allergen-induced Expression of Eotaxin mRNA in the Guinea Pig Lung", pages 1211-1216.	1-12, 14-16, 18, 19

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/06260

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-12, 14-16, 18, and 19

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/06260

## A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

C12N 15/19, 15/63, 5/10, 1/15, 1/21; C07K 14/52; A61K 38/19, 35/00; G01N 33/50

## A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

536/23.5; 435/69.5, 320.1, 240.2, 252.3, 254.11, 7.21, 29; 530/351, 395; 424/85.2; 514/2

## BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

I. Claims 1-12, 14-16, 18, and 19, directed to a  $\beta$ -9 polypeptide, DNA encoding it, and therapeutic and diagnostic methods employing it.

II. Claims 13 and 17, directed to a  $\beta$ -9 antagonist and therapeutic methods employing it.

III. Claim 14, directed to an agonist of  $\beta$ -9 activity (other than the  $\beta$ -9 chemokine itself).

IV. Claim 20, directed to a screening assay using cells expressing a receptor for  $\beta$ -9.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons.

The special technical feature of Group I which defines an advance over the art is the novel  $\beta$ -9 polypeptide. The special technical feature of Group II is a compound which antagonizes the biological activity of  $\beta$ -9. This group does not share the special technical feature of Group I because no embodiment of Group II is characterized by the polypeptide structure of  $\beta$ -9, nor is any embodiment materially or functionally related to any  $\beta$ -9 polypeptide or nucleic acid. Likewise, the special technical feature of Group III is a  $\beta$ -9 antagonist, and this group does not share the special technical feature of Group I as no embodiment is characterized by or related in a material or functional manner to the  $\beta$ -9 polypeptide of the latter group. Group IV does not share the special technical feature of Group I because its special technical feature is modulation of the receptor for  $\beta$ -9 in a system specifically adapted for the detection of such modulation. Although it responds to binding by  $\beta$ -9, the receptor for this chemokine is a materially and functionally separate entity, and the method of Group IV does not require the  $\beta$ -9 chemokine of Group I for its practice.

As noted above, the special technical feature of Group II is a compound which antagonizes the activity of  $\beta$ -9. Group III does not share this special technical feature because its special technical feature is an antagonist which will be a materially and functionally separate entity having functional properties opposing those of the agonist of Group II. Group IV also does not share the special technical feature of Group II because the assay of that group is characterized by an unrelated advance over the art, viz., a specific receptor for  $\beta$ -9 which has been placed in a system specifically adapted for the detection of its modulation. The method of Group IV as claimed does not require the compound of Group III for its practice.

Finally, Groups III and IV do not share a special technical feature: that of the former is a compound which specifically antagonizes the activity of  $\beta$ -9, and of the latter, a specific receptor for  $\beta$ -9 which has been placed in a system specifically adapted for the detection of its modulation. The method of Group IV as claimed does not require the compound of Group II for its practice.

For these reasons, the inventions are not considered to be so linked in any pairing by a special technical feature so as to form a single general inventive concept within the meaning of PCT Rule 13.1.

ID AAT18015  
 CS 95e8244a5f5a364f7626baf6ed7f00cd  
 IDH AAT18015 standard; cDNA; 405 BP.  
 MO DNA  
 DV DNA  
 DT1 01-OCT-1996 (first entry)  
 DT 01-OCT-1996  
 AK PATENT WO9606169-A1  
 AK PRIMARY AC AAT18015  
 EAK  
 DR P-PSDB AAR81567  
 DR WPI 1996-151372/15  
 EDR  
 DE Chemokine beta-9 coding sequence.  
 KW Ck beta-9  
 KW Human  
 KW IL-2 biosynthesis  
 KW autoimmune disease  
 KW bone marrow stem cell colony formation  
 KW breast lymph node  
 KW cancer chemotherapy  
 KW chemokine  
 KW chemokine beta-9  
 KW eotaxin  
 KW epidermal keratinocyte proliferation  
 KW inhibition  
 KW leukaemia  
 KW psoriasis  
 KW solid tumour  
 KW ss  
 EKW  
 OS Homo sapiens.  
 SC 4a22b9fcfbf65eca55a08e9eb4312eae  
 SP HOMO SAPIENS  
 ESP  
 INST (HUMA-) HUMAN GENOME SCI INC  
 CC This sequence encodes human chemokine beta-9 (Ck beta-9). The  
 CC polynucleotide encoding CK beta-9 was discovered in a cDNA library  
 CC derived from a human breast lymph node. Ck beta-9 is structurally  
 CC related to the chemokine family. It exhibits the highest degree of  
 CC homology to eotaxin with 32% identity and 69% similarity over a  
 CC stretch of 75 amino acids. The four spatially conserved cysteine  
 CC residues found in chemokines are also found in Ck beta-9. Ck beta-9  
 CC polypeptides may be used to inhibit bone marrow stem cell colony  
 CC formation as adjunct protective treatment during cancer chemotherapy and  
 CC for leukaemia. They can also be used to inhibit epidermal keratinocyte  
 CC proliferation for treatment of psoriasis. They may be used to treat  
 CC solid tumours by stimulating the invasion and activation of host defence  
 CC cells, e.g. cytotoxic T cells and macrophages. They act to enhance host  
 CC defences against resistant chronic infections, and to treat autoimmune  
 CC disease and lymphocytic leukaemia by inhibiting T cell proliferation by  
 CC the inhibition of IL-2 biosynthesis.  
 ECC  
 RN 1 bfa531145dbf0ef207e572999df71a11  
 RC PD: 29-FEB-1996. PF: 06-JUN-1995; 95WO-US06260. PR: 23-AUG-1994; 94US-  
 0294251.  
 RT Human chemokine beta-9 polynucleotide(s), polypeptide(s) and antagonists  
 - useful in treatment of e.g. leukaemia, tumours, chronic infections and auto-  
 immune disorders and diagnosis of Ck beta-9 mutation(s)  
 RL Patent: WO9606169-A1. Claim 1; Page 40; 55pp; English.  
 RA Adams M.D. ADAMS MD  
 RA Li H. LI H  
 ERN  
 FK sig\_peptide



```

LOC 1..69
LO 1 P 69 P 1 69 +
FQ note
FQ tag
QD a
NT Leader sequence
EFK
FK mat_peptide
LOC 70..405
LO 70 P 405 P 70 405 +
FQ tag
QD b
EFK
SQH Sequence 405 BP; 102 A; 120 C; 117 G; 66 T; 0 other;
SL 405 09dc0d38084416450f4f610805e15513
SQ atggctcagtcactggctctgagcctccttatcctgggttctggcctttggcatccccagg
SQ acccaaggcagtgatggagggggtcaggactggtgcctcaagtacagccaaaggaagatt
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ESQ
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Query: 1   atggctcagtcactggctctgagcctccttatcctgggtcttgcccttggcatccccagg 60
           |||
Sbjct: 1   atggctcagtcactggctctgagcctccttatcctgggtcttgcccttggcatccccagg 60

Query: 61   acccaaggcagtgatggaggggctcaggactggtgcctcaagtacagccaaaggaagatt 120
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Sbjct: 61   acccaaggcagtgatggaggggctcaggactggtgcctcaagtacagccaaaggaagatt 120

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Query: 181 gctatcctgttcttgccccgcaagcgctctcaggcagagctatgtgcagacccaaaggag 240
|||||
Sbjct: 181 gctatcctgttcttgccccgcaagcgctctcaggcagagctatgtgcagacccaaaggag 240

Query: 241 ctctgggtgcagcagctgatgcagcatctggacaagacaccatccccacagaaaccagcc 300
|||||
Sbjct: 241 ctctgggtgcagcagctgatgcagcatctggacaagacaccatccccacagaaaccagcc 300

Query: 301 cagggctgcaggaaggacaggggggcctccaagactggcaagaaaggaaagggtccaaa 360
|||||
Sbjct: 301 cagggctgcaggaaggacaggggggcctccaagactggcaagaaaggaaagggtccaaa 360

Query: 361 ggctgcaagaggactgagcggtcacagaccctaaaggggcca 402
|||||
Sbjct: 361 ggctgcaagaggactgagcggtcacagaccctaaaggggcca 402

```

Database: Geneseq.NA.2002AUG30  
 Posted date: Sep 4, 2002 11:15 AM  
 Number of letters in database: 1,057,821,503  
 Number of sequences in database: 2,085,897

Lambda      K      H  
       1.37    0.711    1.31

Gapped  
 Lambda      K      H  
       1.37    0.711    1.31

Matrix: blastn matrix:1 -3  
 Gap Penalties: Existence: 5, Extension: 2  
 Number of Hits to DB: 251332  
 Number of Sequences: 2085897  
 Number of extensions: 251332  
 Number of successful extensions: 70613  
 Number of sequences better than 10.0: 88  
 length of query: 402  
 length of database: 1,057,821,503  
 effective HSP length: 20  
 effective length of query: 382  
 effective length of database: 1,016,103,563  
 effective search space: 388151561066  
 effective search space used: 388151561066  
 T: 0  
 A: 0  
 X1: 6 (11.9 bits)  
 X2: 10 (19.8 bits)  
 S1: 12 (24.3 bits)

**ID** **AAR81567**  
**CS** fa2eb3e3646f643971306d01d1c2ba38  
**IDH** AAR81567 standard; Protein; 134 AA.  
**MO** PRT  
**DV** PRT  
**DT1** 01-OCT-1996 (first entry)  
**DT** 01-OCT-1996  
**AK** **PATENT** **WO9606169-A1**  
**AK** PRIMARY AC AAR81567  
**EAK**  
**DR** N-PSDB AAT18015  
**DR** WPI 1996-151372/15  
**EDR**  
**DE** Chemokine beta-9.  
**KW** Ck beta-9  
**KW** Human  
**KW** IL-2 biosynthesis  
**KW** autoimmune disease  
**KW** bone marrow stem cell colony formation  
**KW** breast lymph node  
**KW** cancer chemotherapy  
**KW** chemokine  
**KW** chemokine beta-9  
**KW** eotaxin  
**KW** epidermal keratinocyte proliferation  
**KW** inhibition  
**KW** leukaemia  
**KW** psoriasis  
**KW** solid tumour  
**EKW**  
**OS** Homo sapiens.  
**SC** 4a22b9fcfbf65eca55a08e9eb4312eae  
**SP** HOMO SAPIENS  
**ESP**  
**INST** (HUMA-) HUMAN GENOME SCI INC  
**CC** This sequence represents human chemokine beta-9 (Ck beta-9). The  
**CC** polynucleotide encoding CK beta-9 was discovered in a cDNA library  
**CC** derived from a human breast lymph node. Ck beta-9 is structurally  
**CC** related to the chemokine family. It exhibits the highest degree of  
**CC** homology to eotaxin with 32% identity and 69% similarity over a  
**CC** stretch of 75 amino acids. The four spatially conserved cysteine  
**CC** residues found in chemokines are also found in Ck beta-9. Ck beta-9  
**CC** polypeptides may be used to inhibit bone marrow stem cell colony  
**CC** formation as adjunct protective treatment during cancer chemotherapy and  
**CC** for leukaemia. They can also be used to inhibit epidermal keratinocyte  
**CC** proliferation for treatment of psoriasis. They may be used to treat  
**CC** solid tumours by stimulating the invasion and activation of host defence  
**CC** cells, e.g. cytotoxic T cells and macrophages. They act to enhance host  
**CC** defences against resistant chronic infections, and to treat autoimmune  
**CC** disease and lymphocytic leukaemia by inhibiting T cell proliferation by  
**CC** the inhibition of IL-2 biosynthesis.  
**ECC**  
**RN** 1 15649415ba29211a037734c7bd17deb7  
**RC** **PD:** 29-FEB-1996. **PF:** 06-JUN-1995; 95WO-US06260. **PR:** 23-AUG-

1994; 94US-0294251.

RT Human chemokine beta-9 polynucleotide(s), polypeptide(s) and antagonists  
- useful in treatment of e.g. leukaemia, tumours, chronic infections and auto-immune disorders and diagnosis of Ck beta-9 mutation(s)

RL Patent: WO9606169-A1. Claim 12; Page 41; 55pp; English.

RA Adams M.D. ADAMS MD

RA Li H. LI H

ERN

FK Peptide

LOC 1..23

LO 1 P 23 P 1 23 +

FQ note

NT Leader sequence

EFK

FK Protein

LOC 24..134

LO 24 P 134 P 24 134 +

FQ note

NT Mature Ck beta-9

EFK

SQH Sequence 134 AA;

SL 134 3ee44d4be71ba9437a1527c7b1ad15ae

SQ maqslalsllilvlafgiprtqgsdgggaqdcclkyqrkipakvvrsyrkqepslgcsip

SQ ailflprkrsgaelcadpkelyvqqqlmqhldktpspqkpaqgcrkdrkasktgkkkgkgs

SQ gckrtersqtpkpg

ESQ

NCBI-BLASTP 2.0.10 [Aug-26-1999]

Reference: Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schaffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

Query= PF-0027US\_SEQ ID NO:4\_PANEC-2\_226152  
(134 letters)

Database: Geneseq.AA.2002AUG30  
887,419 sequences; 130,709,309 total letters

Searching.....done

Sequences producing significant alignments:	Score (bits)	E Value
GSEQ:ABG60666 Small inducible cytokine subfamily A member 21	278	1e-74
GSEQ:ABB95552 Human angiogenesis related protein PRO1600 SEQ	278	1e-74
GSEQ:ABB84946 Human PRO1600 protein sequence SEQ ID NO:260.	278	1e-74
GSEQ:AAB99213 Human SLC-interleukin-2 fusion protein #2.	278	1e-74
GSEQ:AAB99208 Human SLC-interleukin-2 fusion protein #1.	278	1e-74
GSEQ:AAB99206 Human SLC.	278	1e-74
GSEQ:AAB50860 Human CKbeta-9.	278	1e-74
GSEQ:AAB31199 Amino acid sequence of human polypeptide PRO160	278	1e-74
GSEQ:AAB01434 Human secondary lymphoid chemokine (SLC).	278	1e-74
GSEQ:AA96923 Human chemokine L105_3.	278	1e-74
GSEQ:AAW87588 A human L105 chemokine designated huL105_3.	278	1e-74
GSEQ:AAW50885 Amino acid sequence of human 6CKine protein.	278	1e-74
GSEQ:AAW69163 CC-type chemokine protein designated SLC.	278	1e-74
GSEQ:AAW00668 Pancreas expressed chemokine-2.	278	1e-74
GSEQ:AAG03773 Human secreted protein, SEQ ID NO: 7854.	276	4e-74
<b>GSEQ:AAR81567 Chemokine beta-9.</b>	274	1e-73
GSEQ:AA96922 Human chemokine L105_7.	269	5e-72
GSEQ:AAW87589 A human L105 chemokine designated huL105_7.	269	5e-72
GSEQ:AA912316 Human 5' EST secreted protein SEQ ID NO:347.	266	4e-71
GSEQ:AAO20021 Human chemokine SLC protein.	236	3e-62
GSEQ:AAO14154 Human SLC protein.	236	3e-62
GSEQ:AAB99211 Murine SLC-interleukin-2 fusion protein.	201	2e-51
GSEQ:AAW50884 Amino acid sequence of mouse 6CKine protein.	199	8e-51
GSEQ:AAW28511 Product of clone L105.	199	8e-51
GSEQ:AAB99209 Murine SLC.	198	1e-50

>GSEQ:AAR81567 Chemokine beta-9.

Length = 134

Score = 274 bits (694), Expect = 1e-73

Identities = 133/134 (99%), Positives = 134/134 (99%)

Query: 1 MAQSLALSLLILVLAFGIPRTQGSDDGAQDCCLKYSQRKIPAKVVRSYRKQEPSLGCSIP 60  
MAQSLALSLLILVLAFGIPRTQGSDDGAQDCCLKYSQRKIPAKVVRSYRKQEPSLGCSIP  
Sbjct: 1 MAQSLALSLLILVLAFGIPRTQGSDDGAQDCCLKYSQRKIPAKVVRSYRKQEPSLGCSIP 60

Query: 61 AILFLPRKRSQAELCADPKELWVQQLMQHLDKTPSPQKPAQGCRKDRGASKTGKKGKGSK 120  
AILFLPRKRSQAELCADPKEL+WVQQLMQHLDKTPSPQKPAQGCRKDRGASKTGKKGKGSK  
Sbjct: 61 AILFLPRKRSQAELCADPKELYVQQLMQHLDKTPSPQKPAQGCRKDRGASKTGKKGKGSK 120

Query: 121 GCKRTERSQTPKGP 134  
GCKRTERSQTPKGP  
Sbjct: 121 GCKRTERSQTPKGP 134

Database: Geneseq.AA.2002AUG30  
Posted date: Sep 4, 2002 9:25 AM  
Number of letters in database: 130,709,309  
Number of sequences in database: 887,419

Lambda	K	H
0.316	0.133	0.400

Gapped

Lambda	K	H
0.270	0.0470	0.230

Matrix: BLOSUM62  
Gap Penalties: Existence: 11, Extension: 1  
Number of Hits to DB: 37722616  
Number of Sequences: 887419  
Number of extensions: 1508406  
Number of successful extensions: 6373  
Number of sequences better than 10.0: 1105  
Number of HSP's better than 10.0 without gapping: 438  
Number of HSP's successfully gapped in prelim test: 667  
Number of HSP's that attempted gapping in prelim test: 5016  
Number of HSP's gapped (non-prelim): 1156  
length of query: 134  
length of database: 130,709,309  
effective HSP length: 51  
effective length of query: 83  
effective length of database: 85,450,940  
effective search space: 7092428020  
effective search space used: 7092428020  
T: 11  
A: 40  
X1: 16 ( 7.3 bits)  
X2: 38 (14.8 bits)  
X3: 64 (24.9 bits)  
S1: 41 (21.6 bits)